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Rapid and Sensitive Detection of Shiga Toxin-Producing *Escherichia coli* from Nonenriched Stool Specimens by Real-Time PCR in Comparison to Enzyme Immunoassay and Culture▽

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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) bacteria are a frequent cause of food-borne gastroenteritis, hemorrhagic colitis, and hemolytic uremic syndrome. Because antimicrobial agents are generally contraindicated in patients infected with STEC, a sensitive and specific diagnostic test with rapid turnaround is essential. Current culture methods may fail to detect non-O157 STEC. We evaluated a Stx gene real-time PCR assay using hybridization probes and the LightCycler instrument with 204 prospectively collected stool specimens, which were also tested for Stx by enzyme immunoassay (EIA) (ProSpecT STEC; Remel, Lenexa, KS) and by culturing on chromogenic agar (Chromagar O157; BD BBL, Sparks, MD). In addition, 85 archived stool specimens previously tested for Stx (by EIA) and/or *E. coli* O157:H7 (by culture) were tested by PCR. Sample preparation for PCR included mixing the stool in sterile water and extraction of nucleic acid using the MagNA Pure LC instrument (Roche Diagnostics). The PCR assay had 100% sensitivity and specificity compared to EIA and culture for specimens collected prospectively (4 of 204 specimens were positive) and compared to culture and/or EIA for archival specimens (42 of 85 specimens were positive). Both the EIA and PCR produced positive results from a specimen containing an O103 serotype STEC in the prospective specimens, and the PCR test detected three positive specimens that contained nonviable STEC in the archived specimens. The PCR assay demonstrated 100% sensitivity and specificity compared to EIA and/or culture and more rapid turnaround than either EIA or culture.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is a frequent cause of food-borne outbreaks of diarrhea (15). Disease caused by STEC is characterized by abdominal pain and bloody diarrhea, and 5 to 15% of those individuals infected with serotype O157:H7 develop hemolytic uremic syndrome (HUS), a potentially life-threatening condition consisting of hemolytic anemia, thrombocytopenia, and kidney failure caused primarily by Stx (8). STEC may carry genes for one or both types of Stx, Stx1 and Stx2 (17).

Although STEC strains are a diverse group of pathogens, up to the present, the most common serotype in the United States has been O157:H7. A common association is that of *E. coli* O157:H7 contaminating ground beef (3, 7), but recent large outbreaks have involved a variety of other foods, including leafy greens (6, 29). The diversity of potentially contaminated food means that patients may acquire STEC infection from many foodstuffs, far beyond the stereotypical risk of undercooked ground beef. The common denominator of tainted food products seems to be direct or indirect contamination from bovine feces. To best detect infected patients and potential outbreaks, clinical laboratories must have tools to quickly and accurately detect STEC in stool specimens. Culture on sorbitol MacConkey agar is an inexpensive, effective, and widely used method based on lack of sorbitol fermentation by *E. coli* O157:H7. Several drawbacks limit the utility of culture, including slow turnaround, false-negative results in antibiotic-treated patients, and false-negative results due to emerging serotypes of non-O157 STEC that ferment sorbitol (1, 14, 16, 29). Alternatively, a method that is increasingly utilized is detection of Stx antigen from stool, either directly or after broth enrichment. Our experience concurs with enzyme immunoassay (EIA) product insert data that optimal sensitivity and specificity are achieved only when a broth enrichment step is employed; this results in slow turnaround.

Here, we describe a real-time PCR assay that can detect STEC using nucleic acid extracts of stool specimens. We evaluated the performance of this assay using both archived stool specimens and prospectively collected specimens and compared the results to those of culture and Stx antigen detection.

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MATERIALS AND METHODS

PCR assay. The PCR assay detects both the stx_1 and stx_2 genes by using primers designed for each of the genes. The master mixture (15 μl) containing 1× Roche LC FastStart DNA Master HybProbe (*Taq* DNA polymerase, reaction buffer, deoxyribonucleoside triphosphate mixture with dUTP instead of dTTP, and 10 mM $MgCl_2$), 3 mM $MgCl_2$, and 1× LC PCR primer-probe set (Table 1) (Stx1a, Stx1b, Stx1f, Stx1r, Stx2a, Stx2b, Stx2f, and Stx2r, kit no. 315; TIB MolBiol LLC, Adelphia, NJ) was added to the LightCycler cuvette. Extracted DNA (5 μl) was added to the reaction mixture. The cycling program was as follows: template denaturing at 95°C for 10 min; amplification of the template for 45 cycles of 10 s at 95°C, 15 s at 55°C (single acquisition), and 15 s at 72°C; and detection of the amplified product by melting analysis for 0 s at 95°C, 20 s at 59°C, 20 s at 40°C, ramp of 0.2°C/s, 0 s at 85°C, ramp of 0.2°C (continuous

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TABLE 1. Primers and probes used in the PCR assay[a]

Gene	Name	Sequence (5′ to 3′)
stx_1[b]	Stx1a primer	CAAGAGCGATGTTACGGT
	Stx1b primer	AATTCTTCCTACACGAACAGA
	Stx1f probe[d]	CTGGGGAAGGTTGAGTAGCG
	Stx1r probe[e]	CCTGCCTGACTATCATGGACA
stx_2[c]	Stx2a primer	GGGACCACATCGGTGT
	Stx2b primer	CGGGCACTGATATATGTGTAA
	Stx2f probe[d]	CTGTGGATATACGAGGGCTTGATGTC
	Stx2r probe[e]	ATCAGGCGCGTTTTGACCATCT

[a] Primer-Probe set, kit no. 315 (Tib MolBiol).
[b] The stx_1 target corresponds to positions 2996451 to 2996243 of GenBank accession no. NC_002655 (21).
[c] The stx_2 target corresponds to positions 1352455 to 1352659 of GenBank accession no. NC_002655 (21).
[d] Labeled with fluorescein on the 3′ end.
[e] Labeled with LC640 on the 5′ end and a phosphate on the 3′ end.

acquisition), and 30 s at 40°C. stx_1 was differentiated from stx_2 amplification by melting curve analysis (Fig. 1). Positive and negative controls were included with each run. The assay was run successfully on two models of the LightCycler, 1.2 and 2.0.

Positive-control plasmids. Two positive control plasmids were constructed using the pCR 2.1 TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The sources of the stx_1 and stx_2 sequences were *E. coli* O157:H7 (ATCC 43890) and O157:H7 (ATCC 43889), respectively. The sequences were amplified using primers Stx1a and Stx1b to clone 208 bp of stx_1 and Stx2a and Stx2b to clone 204 bp of stx_2. The sizes of the cloned sequences were confirmed by agarose gel electrophoresis, and the plasmids were purified using the High Pure Plasmid Isolation Kit (Roche Applied Science, Indianapolis, IN). The control plasmids were diluted in Tris-EDTA buffer (pH 8.0) and stored at 4°C.

Stool processing for PCR. A swab was used to transfer a pea-size amount of stool into 1 ml of sterile water. If the specimen was liquid enough to pipette, a 100- to 200-μl sample was mixed into 1 ml of sterile water. The stool-in-water samples were mixed by the use of a vortex apparatus and allowed to settle for at least 2 min. Then, 200 μl of the supernatant was transferred to a sample cartridge for DNA extraction using a MagNA Pure LC Total Nucleic Acid Isolation Kit on a MagNA Pure LC instrument (Roche Diagnostics).

Analytical sensitivity and specificity, and inhibition. Stools sent to the clinical microbiology laboratory for *Clostridium difficile* testing were prepared as described above and used for the sensitivity and inhibition studies. For sensitivity studies, the stool-in-water samples were spiked with dilutions of *E. coli* O157:H7 before extraction. For inhibition studies, the stool-in-water sample was spiked with the plasmid control (final concentration, 100 copies/μl), and the mixture was extracted on the MagNA Pure LC instrument. To determine specificity, primer and probe sequences were used as subjects for a BLAST search on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). In addition, a panel of nucleic acid extracts from organisms found in stool (Table 2) was evaluated with the PCR assay.

Verification of the PCR results. A human subject protocol for clinical specimens included in this study was approved by the institutional review board prior to any testing. The PCR assay was compared to other methods using two sets of samples. The first was a prospective study including 204 consecutive stool specimens that were submitted to the clinical microbiology laboratory for Stx testing by the ProSpecT EIA (ProSpecT STEC; Remel, Lenexa, KS) with broth enrichment. In addition to the EIA, the specimens were cultured on Chromagar O157 (BD BBL, Sparks, MD). Isolates on Chromagar that were mauve were considered presumptive positive and were then confirmed on sorbitol MacConkey agar (BD BBL, Franklin Lakes, NJ). The EIA and culture plates were used in accordance with the manufacturers' instructions. The second set of stool samples included 85 archived clinical samples that had been frozen at −70°C. The set contained normal and diarrheal stools that were previously tested by routine stool culture (using sorbitol MacConkey agar) and/or tested for Stx by EIA (ProSpecT and/or Premier EHEC EIA [Meridian BioScience Cincinnati, OH]).

A positive PCR result was considered concordant with the comparison methods if a positive result was determined by either culture or EIA. Negative PCR results were considered concordant if all of the comparator methods used were negative. Samples with discordant results were retested by the same PCR assay, and nucleic acid extracts were sent to the Minnesota Department of Health (MDH) for analysis by a PCR method that used different primers (20).

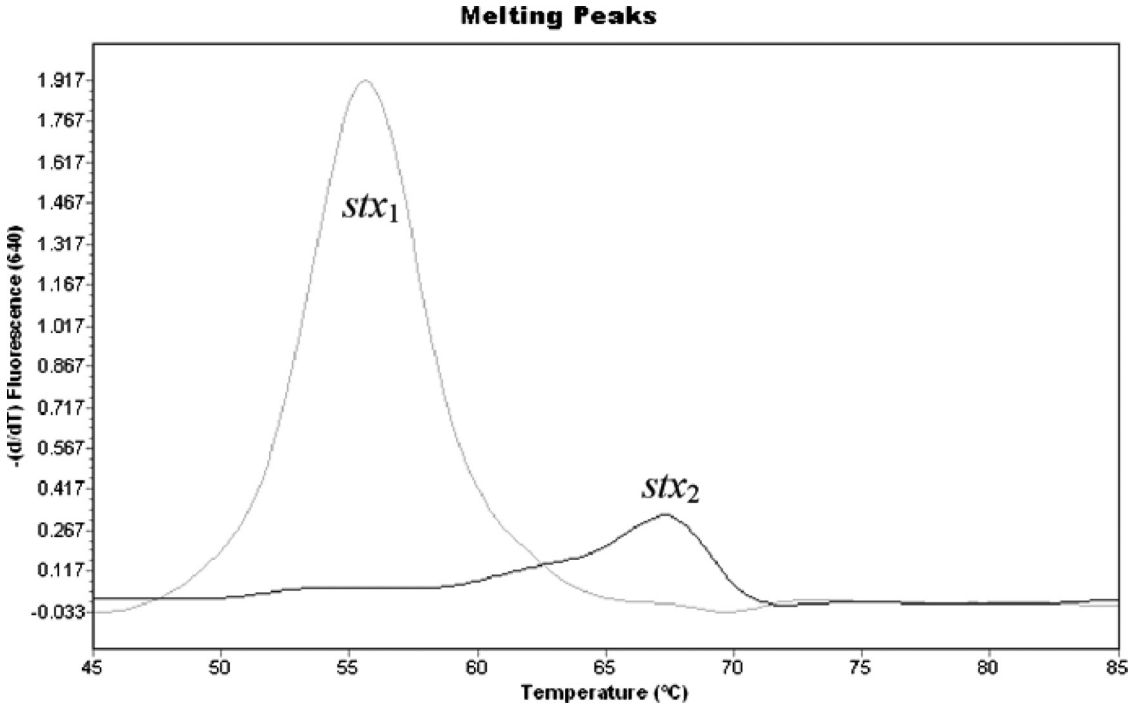

FIG. 1. Melting curve analysis for the PCR assay. Amplification of stx_1 generates a curve with a peak at 55.77°C ± 2°C, while stx_2 generates a peak at 66.91°C ± 2°C. The lower intensity of the stx_2 peak is consistent whether detected alone or with stx_1.

TABLE 2. Organisms tested with the *stx* PCR assay ($n = 66$)

Organism	Accession no. or source[a]
A. haemolyticus	ATCC 17906
A. haemolyticus	ATCC 17977
A. haemolyticus	ATCC 19194
Aeromonas hydrophila	CAP-D-1-82
Arcanobacterium pyogenes	ATCC 19411
Bacteroides distasonis	ATCC 8503
Bacteroides fragilis	ATCC 25285
Bacteroides thetaiotaomicron	ATCC 29741
Bacteroides vulgatus	ATCC 29327
Bifidobacterium adolescentis	ATCC 15703
Bifidobacterium bifidum	ATCC 29521
Bilophila wadsorthia	NYS-01-Ed
Campylobacter coli	ATCC 33559
Campylobacter jejuni	ATCC 33560
C. freundii	ATCC 8090
C. freundii	NYS-2-01
C. freundii	NYS-2003-3 no. 5
C. difficile	ATCC 9689
Clostridium perfingens	ATCC 13124
Clostridium ramosum	ATCC 25582
Collinsella aerofaciens	ATCC 25986
Cryptosporidium sp.	Isolate from cat
Dientamoeba fragilis	ATCC 30948
Eggerthella lenta	ATCC 25559
Encephalitozoon cuniculi	ATCC 50602
Encephalitozoon hellum	ATCC 50451
Encephalitozoon intestinalis	ATCC 50651
Entamoeba histolytica	ATCC 30459
Entamoeba moshkovskii	ATCC 30042
E. cloacae	ATCC 13047
E. cloacae	CAP ID-13-B
E. cloacae	CAP-D-C-no.15
E. cloacae	CAP-D-3-84
Enterococcus faecalis	ATCC 19433-U
Enterococcus faecium	ATCC 19434
E. coli O142:K86(B):H6	ATCC 23985
E. coli O70:K:H42	ATCC 23533
E. coli	ATCC 25922
Escherichia fergusonii	ATCC 35469
Escherichia hermanii	ATCC 33650
Escherichia vulneris	ATCC 33821
Eubacterium rectale	ATCC 33656
Fusobacterium nucleatum	ATCC 25559
Giardia lamblia	ATCC 30957
Klebsiella pneumoniae	ATCC 700603
Lactobacillus delbrueckii subsp. *lacti*	ATCC 12315
Lactobacillus rhamnosus	ATCC 7469
Mycobacterium avium	ATCC 700398
Peptostreptococcus magnus	ATCC 29328
Plesiomonas shigelloides	ATCC 14029
Proteus mirabilis	ATCC 35659
Pseudomonas aeruginosa	ATCC 27853
Salmonella enterica	ATCC 35987
Salmonella group B	CAP-D-1-69
Shigella boydii	ATCC 29903
Shigella dysenteriae	ATCC 25931
S. dysenteriae	CDC 82-002-72
Shigella flexneri	CDC AB4-A04
S. flexneri serotype 2a	ATCC29903
Shigella sonnei	ATCC 25931
S. sonnei	CDC 82-002-72
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 14990
Streptococcus bovis	CAP-D-16-83
Streptococcus sanguinis	ATCC 10556
Yersinia enterocolitica	ATCC 9610

[a] CAP, College of American Pathologists; NYS, New York State.

TABLE 3. Detection of *stx* by PCR in stool compared to a combined standard[a]

Real-time PCR result	No. of prospective specimens		No. of archived specimens		Total no. of specimens	
	Positive[b]	Negative[c]	Positive[d]	Negative[e]	Positive	Negative
Positive	4	0	42	0	46	0
Negative	0	200	0	43	0	243

[a] Discordant results were resolved by a separate PCR method at the MDH. Results that were initially discordant with the real-time PCR results have been recalculated in this table to conform with the second PCR results from the MDH.
[b] One positive by EIA only; three positive by both EIA and culture.
[c] Negative by EIA and culture.
[d] Positive by EIA and/or culture ($n = 39$). Three specimens were negative by EIA and/or culture but positive by the MDH PCR assay.
[e] Negative by EIA and/or culture ($n = 33$). Specimens with discordant results were all negative by the MDH PCR assay ($n = 10$).

RESULTS

The analytical sensitivity of the PCR assay was 100% at 2 copies/μl of extracted specimen or 10 copies/reaction. This is roughly equivalent to 10^4 CFU/g stool specimen. The inhibition studies demonstrated that none of the 53 specimen extracts tested contained substances that would cause a false-negative result in a low-positive sample. Melting curve analysis established that stx_1 melts at 55.77°C ± 2°C, while stx_2 melts at 66.91°C ± 2°C. Fifteen clinical samples yielded peaks between the two ranges. Six of these were sequenced, and all were found to be Stx sequences specific for stx_1 or stx_2. Therefore, a melting peak between 53.77 and 68.91 indicated a positive result for *stx* but did not allow differentiation of stx_1 versus stx_2.

The primers and probes were subjected to BLAST searches to identify potential cross-reacting sequences. Aside from the expected target, there were three organisms with exact matches to the Stx2a primer, *Acinetobacter haemolyticus*, *Enterobacter cloacae*, and *Citrobacter freundii*. At least three isolates of each of these species (strains acquired from ATCC or proficiency specimens from New York State or the College of American Pathologists) were tested in the cross-reactivity studies and were negative. Also, the BLAST search revealed no matches to the Stx2b primer, suggesting that no amplification would result in the event that Stx2a cross-reacted with an unintended sequence. In addition, 56 other organisms found in stool were tested in the assay, and none of them were positive (Table 2).

The accuracy experiments were divided into two parts. One was a series of 204 prospectively collected clinical stool specimens submitted for Stx testing by an EIA method, which we also cultured (Table 3). The other was a set of 85 archived stool specimens, some of which were previously positive for Stx (by EIA) and/or *E. coli* O157:H7 (by culture) and some of which were negative by toxin detection and culture. All of the prospective specimen results were concordant between PCR and EIA, including one positive specimen that was an O103 serotype STEC not detected by culture. Thirteen archived samples produced discordant results: six were previously positive by EIA, three were previously negative by EIA and culture, and four had previously discordant results of culture and EIA. Each of the discordant results was reextracted and retested by PCR. Each of the repeated results was the same as the initial result. To resolve the discordant results, the specimen extracts were sent to the MDH. The MDH tested the samples by

traditional PCR, which used primers for stx_1 and stx_2 different from those in the real-time PCR. The results from the MDH experiments were all concordant with the real-time PCR results. Archived specimen data and combined data from prospective and archived specimens are displayed in Table 3. All initially discordant results were recalculated using the data from MDH and are displayed accordingly in Table 3. The sensitivity and specificity of the assay were both 100% compared to the combined standard.

DISCUSSION

We developed and validated a real-time PCR assay that detects STEC directly from stool with 100% sensitivity and specificity and same-day results, a significant improvement in turnaround versus culture or antigen assays. The PCR assay has the sensitivity to detect as few as 1,000 CFU in a small sample of stool. In addition, two levels of sequence selection impart the high specificity of the assay. The first level involves the primers binding to and amplifying the target sequence. The second level is binding of the hybridization probes to sequences internal to the primer binding sites. Although some of the melting temperatures of the positive samples were between the typical stx_1 and stx_2 ranges, 100% of the positive specimens were detected.

The PCR assay has the ability to differentiate stx_1 and stx_2; however, the significance of this determination has not been resolved. Although some studies have shown that stx_2 tends to be associated with more serious disease (9, 10, 18), stx_1 has also been associated with severe cases. Complicating matters further, some STEC isolates carry genes for both stx_1 and stx_2 or more than one type of stx_2 (10). Notably, either type can cause serious disease, and the treatment for any STEC infection is primarily supportive—antibiotics should be withheld or discontinued (29, 31).

Interestingly, subtypes within the stx_2 group carry different disease risks. The five main subtypes are stx_2, stx_{2c}, stx_{2d}, stx_{2e}, and stx_{2f} (22, 26, 30). The BLAST searches revealed that the primer sequences for this assay matched stx_2 and stx_{2c} (GenBank accession no. Y10775 and M59432, respectively), which are the two types most commonly associated with the postinfection complication HUS, with 100% identity; the other subtypes have rarely or never been associated with HUS. The primer sequences also matched stx_{2d} (GenBank accession no. L11078), found commonly in non-O157:H7 strains, with 100% identity; this subtype is associated with diarrhea but rarely with HUS (23). Subtypes stx_{2e} and stx_{2f} (GenBank accession no. X81416 and M29153, respectively) matched the primer sequences for the assay with less than 90% identity, but these two subtypes are present in seldom-encountered serogroups of *E. coli* that are not typically human pathogens (10, 11, 24).

The PCR assay has the ability to detect non-O157 STEC pathogens, as well as culture-negative stools. Although non-O157 STEC strains are thought to be infrequent in the United States, culture methods using sorbitol MacConkey agar alone may not detect them, so their prevalence may be underestimated (16). Some states have found that non-O157 serotypes cause a substantial portion of STEC cases (5, 12, 19), and many studies from Europe have revealed that O157 serotypes are often the minority of STEC strains detected in diarrheal stools (2, 14). Of the 204 prospective specimens included in this study, one of four positive results was due to a non-O157 serogroup, a rate of 25%. The isolate was an O103 serotype that fermented sorbitol and would have been missed if culture alone had been used. STEC was also detected by the PCR assay in five archived specimens that had been negative by culture (three of which were also negative by EIA). One limitation of the PCR method is that there is no isolate to characterize. Culture isolates are vital to public health efforts to detect outbreaks and track the epidemiology of STEC organisms.

One problem with Stx antigen assays is that a false positive may set off an inappropriate and potentially expensive investigation (4, 12). An additional benefit of the real-time PCR method described here is that the two sets of oligonucleotide primers and probes allow excellent selection of the desired target and minimize false-positive results. This was exemplified by the 100% specificity determined in this study. Another crucial aspect of the real-time platform is the ability to amplify and detect nucleic acid in a closed system, which minimizes the potential for contamination and false positives.

On the other hand, a false-negative result can be equally troubling, as a patient may receive antibiotic treatment, which could increase the risk of HUS. Some previous reports of PCR assays for Stx genes that use stool have demonstrated significant inhibition, and it was found necessary to use either a diluted extract or an enriched specimen (13). The assay described here found a 0% inhibition rate from stool, suggesting that false-negative results will be rare. Our laboratory has previously demonstrated 0% inhibition for 92 stools when they were prepared in the same manner for a *C. difficile* real-time PCR assay (27). In addition, the fact that the same extraction methodology preparatory to PCR can be used on whole blood without significant inhibition suggests that the presence of blood in stool will not impart a risk of false negativity (28).

Our assay is, to the best of our knowledge, the first comparison to culture and EIA of a LightCycler real-time PCR assay using a nonenriched stool specimen for STEC detection. Other groups have designed assays to detect STEC, either real-time PCR from isolated colonies or an enrichment broth or traditional PCR directly from a stool specimen, neither of which significantly improves turnaround time compared to culture or EIA. Schuurman et al. (25) described two real-time assays using stool specimens that were validated with a panel of STEC isolates, a group of 19 previously positive stools, and prospective specimens (3 of 115 stools were positive and confirmed by culture). Our assay compared favorably to the limit of detection but, in contrast, detected no PCR inhibition. We were able to include more than twice as many stool specimens, and our gold standard was a composite of both EIA and culture. Our assay will provide same-day turnaround with excellent sensitivity and specificity, which will provide the fastest possible result to the clinician.

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